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ASSESSMENT OF ANALGESIC AND ANTIBACTERIAL ACTIVITY OF *PREMNA INTEGRIFOLIA* LINN. (FAMILY: VERBENACEAE) LEAVES

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ABSTRACT

Premna integrifolia Linn. (Family: Verbenaceae) is a medicinal plant which is traditionally used against a number of diseases including inflammatory condition. On the scientific basis of traditional uses, the ethanolic extract of leaves of *Premna integrifolia* Linn. was assessed for its possible analgesic and antimicrobial activity. The analgesic activity of the sample was studied using acetic acid induced writhing model in mice. Antibacterial activity of leaves of *Premna integrifolia* Linn. was tested by using the disc diffusion method. In analgesic activity, the extract produced 52.17% ($p<0.01$) acetic acid induced writhing inhibition in mice at the dose of 500 mg/kg body weight, which is comparable to diclofenac sodium 65.21% ($p<0.01$) at the dose of 25 mg/kg body weight. The extract showed significant antibacterial activity against both gram positive and gram negative bacteria. All the results tend to justify the traditional uses of the plant and require further investigation to identify the chemicals responsible for these effects.

INTRODUCTION: *Premna integrifolia* Linn. is a large shrub or a small tree with sub-cordate-ovate, opposite leaves, small greenish or yellowish white flowers in cymes, grows naturally and commonly planted in Sundarban and Chittagong. It is commonly known as ganiari, gambari, bhutbirabi [1].

Stem bark contains tannin, bitter alkaloids, premnine, ganiarine and ganikarine, reducing sugars and unsaturated hydrocarbons. Leaves contain verbascoside, vervascoside iridoid glucoside conjugate, premcoryoside and three monoacyl-6-O-alpha-L-rhamnopyranosylcatalpols [2]. Two new phenolic diterpenoids have been isolated from the root bark [3].

Decoction of root is cordial, stomachic, carminative, alterative, tonic and good for liver complaints, and also useful in urticaria. Leaves are used in fever, colic, flatulence, gonorrhea, convulsion, neuralgia and rheumatism. Extract of stem bark decreases force of contraction of heart and produces dilation of pupils [3].

MATERIALS AND METHODS:

Sample collection and extraction: The leaves of *Premna integrifolia* Linn. were collected from the road side area of Dinajpur, Bangladesh in June, 2009 and identified by the experts of Bangladesh National Herbarium, Mirpur, Dhaka (Accession No. DACB-34546). A voucher specimen has been deposited in Pharmacy Discipline, Khulna University, Khulna- 9208,

Bangladesh. The identified leaves were dried under shade. After complete drying, the leaves were cut into small pieces and then slashed to coarse powder with the help of mechanical grinder and the powder was stored in a suitable container. About 500 gm of powder was extracted by maceration over 20 days with 1200 ml of 80% ethanol. The whole mixture then underwent a coarse filtration by a piece of clean, white cotton material. Then it was filtered through filter paper. The filtrate thus obtained was evaporated by using a rotary evaporator to get a viscous mass. The viscous mass was then vaccum dried to get a dried ethanolic extract (approx. yield value 10%). The extract thus obtained was used for experimental purposes.

Animals: Swiss-Albino mice of either sex (20-25 gm body weight) were taken from animal resources branch of the "International Center for Diarrhoeal Disease Research, Bangladesh" (ICDDR, B) and were used for the experiments. The animals were kept in the standard polypropylene cages and provided with standard diets (ICDDR, B formulated). The animals were acclimatized in animal house, Pharmacy Discipline, Khulna University, Khulna under standard Laboratory conditions (relative humidity 55-60%, room temperature 25±2^{o}C and 12 hours light: dark cycle) for period of 14 days prior to performing the experiments [4].

Microorganisms: Ten (10) pathogenic bacterial strains both gram positive and gram negative were collected from the "International Center for Diarrhoeal Disease Research, Bangladesh" (ICDDR, B).

Drug: The standard drug Diclofenac sodium was collected from Beximco Pharmaceuticals Ltd. Dhaka, Bangladesh.

Phytochemical tests: The crude extract was subjected to preliminary phytochemical screening for the detection of major functional groups [5]. Then, the extract was used for pharmacological screening.

The reagents used for the different chemical groups test: The following reagents were used for the different chemical groups test.

Mayer's reagent: 1.36 gm mercuric iodide in 60 ml of water was mixed with a solution contains 5 gm of potassium iodide in 20 ml of water.

Dragendroff's Reagent: 1.7 gm basic bismuth nitrate and 20 gm tartaric acid ware dissolved in 80 ml water. This solution was mixed with a solution contains 16 gm potassium iodide and 40 ml water.

Fehling's solution A: 34.64 gm copper sulphate was dissolved in a mixture of 0.50 ml of sulfuric acid and sufficient water to produce 500 ml.

Fehling's solution B: 176 gm of sodium potassium tartarate and 77 gm of sodium hydroxide were dissolved in sufficient water to produce 500 ml equal volume of above solution was mixed at the time of use.

Benedicts Reagent: 1.73 gm cupric sulphate, 1.73 gm sodium citrate and 10 gm anhydrous sodium carbonate were dissolved in water and the volume was made up to 100 ml with water.

Molisch Reagent: 2.5 gm of pure α-naphthol was dissolved in 25 ml of ethanol.

Liebermann- Burchard Reagent: 5 ml acetic anhydride was carefully mixed under cooling with 5ml concentrated sulfuric acid. This mixture was added cautiously to 50 ml absolute ethanol with cooling.

Tests procedure for identifying different chemical groups: The following tests were performed for identifying different chemical groups [3, 5].

Tests for reducing sugar:

Benedict's test: 0.5 ml of aqueous extract of the plant material was taken in a test tube. 5ml of Benedict's solution was added to the test tube, boiled for 5 minutes and allowed to cool spontaneously. A red color precipitate of cuprous oxide was formed in the presence of a reducing sugar.

Fehling's Test (Standard Test): 2ml of an aqueous extract of the plant material was added 1ml of a mixture of equal volumes of Fehling's solutions A and B. Boiled for few minutes. A red or brick red color precipitate was formed in the presence of a reducing sugar.

Test for tannins:

Ferric Chloride Test: 5 ml solution of the extract was taken in a test tube. Then 1 ml of 5%Ferric chloride solution was added. Greenish black precipitate was formed and indicated the presence of tannins.

Potassium dichromate test: 5 ml solution of the extract was taken in a test tube. Then 1 ml of 10% Potassium dichromate solution was added. A yellow precipitate was formed in the presence of tannins.

Test for Flavonoids: Added a few drops of concentrated hydrochloric acid to a small amount of an alcoholic extract of the plant material. Development of a red color was indicated the presence of Flavonoids.

Test for Saponins: 1 ml solution of the extract was diluted with distilled water to 20 ml and shaken in a graduated cylinder for 15 minutes. No layer of foam was indicated the absence of saponins.

Test for Gums: 5 ml solution of the extract was taken and then Molish reagent and sulphuric acid were added. No red violet ring produced at the junction of two liquids indicated the absence of gums.

Test for Steroids:

Liebermann-Burchard test: 1ml solution of chloroform extract was taken and then added 2 ml Libermann-Burchard reagent. Reddish purple color indicated the presence of steroids.

Sulphuric acid test: 1 ml solution of chloroform extract was taken and then added1ml Sulphuric acid. Red color indicated the presence of steroid.

Test for alkaloids:

Mayer's test: 2 ml solution of the extract and 0.2 ml of dilute hydrochloric acid were taken in a test tube. Then 1 ml of Mayer's reagent was added. Yellow color precipitate was formed and that was indicated as the presence of alkaloids.

Dragendroff's test: 2 ml solution of the extract and 0.2 ml of dilute hydrochloric acid were taken in a test tube. Then 1 ml of Dragendroff's reagent was added. Orange brown precipitate was formed and that was indicated as the presence of alkaloids.

Determination of Analgesic Activity: The analgesic activity of the sample was studied using acetic acid induced writhing model in mice. 1% Tween-80 was used as solvent. Experimental animals were randomly selected and divided into four groups denoted as Control group, Positive control group and Test group I and Test group II consisting of five (05) mice in each group. Control group received orally 1% Tween-80 at the dose of 10 mg/kg body weight and Positive control group received orally diclofenac sodium at the dose of 25 mg/kg body weight.

Test group I and Test group II were treated with test sample orally at the dose of 250 and 500 gm/kg body weight. A thirty minutes interval was given to ensure proper absorption of the administered substances. Then the writhing inducing chemical, acetic acid solution (0.7%) was administered intra-peritoneally to each of the animals of a group. After an interval of 5 minutes was given for absorption of acetic acid and number writhing was counted for 15 minutes. The animals do not always perform full writhing. The incomplete writhing was taken as half-writhing, so two half-writhing were taken as one full writhing. This is why total writhing was halved to convert all writhing to full writhing or real writhing.[6, 7]

Determination of Antibacterial Activity: Antibacterial activity of leaves of *Premna integrifolia* Linn. was tested by using the disc diffusion method [8, 9]. Nutrient agar media was used. Sample impregnated discs, standard antibiotic discs (Kanamycin) and negative control discs were placed gently on the seeded agar plates with the help of sterile forceps to assure complete contact with medium surface.

The plates were then inverted and kept in refrigeration for about 2 h at 4°C to allow the material to diffuse into a considerable area of the medium. Finally the plates were incubated upside down at 37°C for 24 h. After proper incubation, the antibacterial activity of the test agent was determined by measuring the diameter of zone of inhibition in terms of millimeter with a slide calipers.

Statistical analysis: Student's t-test was used to determine significant differences between the control group and test group.

RESULTS: In the preliminary phytochemical screening the extract showed the presence of reducing sugars, tannins, glycosides, alkaloids, flavonoids, and steroids (**Table 1**).

TABLE 1: RESULTS OF PRELIMINARY PHYTOCHEMICAL ANALYSIS

Plant Extract	Alkaloids	Glycosides	Steroids	Gums	Tannins	Saponins	Flavonoids	Reducing sugar
Ethanolic extract of *Premna integrifolia* Linn.	+	+	+	-	+	-	+	+

+ = Presence; - = Absence

Analgesic activity test: Analgesic activity of the ethanolic extract of leaves of *Premna integrifolia* Linn. was tested by acetic acid induced writhing model in mice. The extract produced 52.17% ($p<0.01$) acetic acid induced writhing inhibition in mice at the dose of 500 mg/kg body weight, which is comparable to diclofenac sodium 65.21% ($p<0.01$) at the dose of 25 mg/kg body weight (**Table 2**).

TABLE 2: EFFECT OF LEAVES OF *PREMNA INTEGRIFOLIA* LINN. ON ACETIC ACID INDUCED WRITHING IN MICE

Animal Group	Treatment	Writhing Count (%Writhing)	%Writhing Inhibition
Control (n=5)	1% tween-80 solution in water	4.6 ± 1.17 (100)	0
Positive Control (n=5)	Diclofenac sodium (25mg/kg)	1.6 ± 0.74* (34.79)	65.21
Test group I (n=5)	Et. Extract (250mg/kg)	2.8 ± 0.45* (60.87)	39.13
Test group I (n=5)	Et. Extract (500mg/kg)	2.2 ± 0.5.1* (47.83)	52.17

Values are expressed as mean ± SEM, SEM=Standard error of Mean, n=No. of mice, Et. = Ethanolic, **P* < 0.01 vs. control

Antibacterial activity test: Table 3 showed the results of antibacterial test. The antibacterial activity was assessed against a panel of 10 pathogenic bacterial strains (Both gram positive and gram negative) at the dose of 250 and 500 μg/disc, and the results (zone of inhibition) were compared with the activity of the positive control, kanamycin (30 μg/disc). At 250 μg/disc, the extract showed average zone of inhibition against *Streptococcus pyogenes* (6 mm), *Enterococcus faecalis* (5 mm), *Streptococcus agalactiae* (7 mm), *Shigella boydii* (4 mm), *Shigella flexneri* (6 mm), *Shigella dysenteriae* (8 mm), *Pseudomonas aeruginosa* (8 mm), *Shigella sonnei* (6 mm) and *Escherichia coli* (5 mm). At 500 μg/disc, the extract showed average zone of inhibition against *Streptococcus pyogenes* (12 mm), *Enterococcus faecalis* (9 mm), *Streptococcus agalactiae* (10 mm), *Shigella boydii* (9 mm), *Shigella flexneri* (10 mm), *Shigella dysenteriae* (14 mm), *Pseudomonas aeruginosa* (15 mm), *Shigella sonnei* (11 mm) and *Escherichia coli* (12 mm).

TABLE 3: ANTIBACTERIAL ACTIVITY OF ETHANOLIC EXTRACT LEAVES OF *PREMNA INTEGRIFOLIA* LINN.

Bacterial Strains	Type of Bacterial Strains	Diameter of Zone of Inhibition in mm			
		Blank	Kanamycin (μg/disc)	Extract (250μg/disc)	Extract (500μg/disc)
Streptococcus pyogenes	Gram(+)	-	18	6	12
Enterococcus faecalis	Gram(+)	-	15	5	9
Streptococcus agalactiae	Gram(+)	-	20	7	10
Staphylococcus saprophyticus	Gram(+)	-	27	-	-
Shigella boydii	Gram(-)	-	20	4	9
Shigella flexneri	Gram(-)	-	26	6	10
Shigella dysenteriae	Gram(-)	-	20	8	14
Pseudomonas aeruginosa	Gram(-)	-	15	8	15
Shigella sonnei	Gram(-)	-	10	6	11
Escherichia coli	Gram(-)	-	21	5	12

Gram (-): Gram Negative Bacteria; Gram (+): Gram Positive Bacteria; (-): No inhibition

DISCUSSION: To get preliminary idea about the active constituents present in the leaves extract different chemical tests were performed and found the presence of Reducing sugar, Tannins, Flavonoids, Steroids, Alkaloid, Glycosides. Analgesic activity of the ethanolic extract of *Premna integrifolia* Linn. was tested by acetic acid induced writhing model in mice. The writhing test is generally used for screening of

antinociceptive effects [16]. With respect to the writhing test, the research group of Deraedt *et al.* (1980), described the quantification of prostaglandins by radioimmunoassay in the peritoneal exudates of rats, obtained after intraperitoneal injection of acetic acid.

Acetic acid induced writhing model represents pain sensation by triggering localized inflammatory response. Acetic acid, which is used to induce writhing, causes algesia by liberation of endogenous substances, which in turn excite the pain nerve endings.[10] Increased levels of PGE_2 and $PGF_{2\alpha}$ in the peritoneal fluid have been reported to be responsible for pain sensation caused by intraperitoneal administration of acetic acid [11]. The ethanolic extract of *Premna integrifolia* Linn. produced significant writhing inhibition comparable to the standard drug diclofenac sodium. On the basis of this result it can be concluded that the ethanol extract of *Premna integrifolia* Linn. possesses analgesic activity.

Antibacterial activity was tested by using the disc diffusion method. Disc diffusion method is widely acceptable for the preliminary screening of antibacterial activity. It is essentially a qualitative or semi qualitative test indicating the sensitivity or resistance of microorganisms to the test materials [15]. The antibacterial activity was assessed against a panel of 10 pathogenic bacterial strains (both gram positive and gram negative) at the dose of 250 and 500 μg/disc and the results were compared with the activity of the positive control, kanamycin (30 μg/disc). The extract was found active against both gram positive and gram negative bacteria except *Staphylococcus saprophyticus* and the inhibitory effects on tested species showed dose dependence.

The zone of inhibition varies within the ranges of 4-8 mm and 9-15 mm at the dose of 250 and 500 μg/disc respectively. The highest zone of inhibition was found against *Pseudomonas aeruginosa* (15 mm) and showed moderate activity against *Streptococcus pyogenes, Shigella dysenteriae*, *Shigella sonnei* and *Escherichia coli*. The results support the traditional use of this plant as a remedy of infectious diseases like diarrhea, dysentery, shigellosis and gastrointestinal disturbances.

CONCLUSION: According to above discussion, *Premna integrifolia* Linn. contains important chemical constituents that confer upon it as a medicinal agent. It was revealed that the leaf extract contains Reducing Sugar, Tannins, flavonoids, Steroids, Alkaloids, and Glycosides which have potential role in its Analgesic and Antimicrobial activity. This could provide a rationale for traditional uses of this plant and suggests for further investigation and isolation of biologically active constituents responsible for the activity.

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